NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

INCORPORATION OF ANTIBIOTICS EFFECTIVE AGAINST MULTIDRUG-RESISTANT PATHOGENS INTO PMMA FOR CRANIO-MAXILLOFACIAL IMPLANTS

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BIOMATERIALS AND EPIDEMIOLOGY
CRANIOFACIAL HEALTH AND RESTORATIVE MEDICINE

NAMRU-SA REPORT # 2018-02

DECLARATION OF INTEREST

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ACKNOWLEDGEMENTS

This work was funded by the Naval Medical Research Center's Advanced Medical Development Program using work unit number G1315. This research was also supported in part by an appointment to the Postdoctoral Research Participation Program at the Naval Medical Research Unit San Antonio (NAMRU-SA) administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and NAMRU-SA.

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ABBREVIATIONS

µL	Microliter
A. baumannii	*Acinetobacter baumannii*
ASTM	American Society for Testing and Materials
ATCC	American tissue cell culture
C	Colistin
CFUs	Colony forming units
DSC	Differential scanning calorimetry
g	Gram
GPC	Gas permeation chromatography
HPLC	High performance liquid chromatography
ISO	International standardization organization
M	Minocycline
MDR	Multidrug resistant
mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
mL	Milliliter
mm	Millimeter
MMA	Methyl methacrylate
M_n	Number average molecular weight
MPa	Megapascal
MS/MS	Multiple steps/Mass spectrometry
M_w	Weight average molecular weight
OD	Optical density
PB	Polymyxin B
pH	Power of Hydrogen
PMMA	Polymethyl methacrylate
S. aureus	*Staphylococcus aureus*
T_g	Glass transition temperature
UV	Ultraviolet

EXECUTIVE SUMMARY

Background: The rise in incidence of infections caused by multidrug-resistant (MDR) pathogens such as *Staphylococcus aureus* and *Acinetobacter baumannii*, presents a problem in the treatment of post-surgical infections at cranio-maxillofacial implant sites. Incorporation of antibiotics against MDR pathogens into polymethyl methacrylate (PMMA) is a potential treatment to obtain a favorable outcome for those with cranio-maxillofacial defects.

Objective: Evaluate the structural, mechanical, and antimicrobial activity of PMMA incorporated with colistin, polymyxin B, or minocycline against Gram-positive *S. aureus* and Gram-negative *A. baumannii*.

Methods: Polymethyl methacrylate rectangular rods incorporated with antibiotics colistin, polymyxin B, or minocycline were fabricated by adding the antibiotics at concentrations of 0.5g and 1.0g to Lucite acrylic resin and cured for 8 hours at 71.1°C. Gas chromatography was used to determine the molecular weight and the amount of residual monomer of the antibiotic incorporated PMMA rods. Mechanical properties of the rods were determined by differential scanning calorimetry, compression and flexural testing. Antibiotic elution was determined for each antibiotic by using HPLC with UV detection, and samples were taken at time points beginning with one hour up to 30 days. The eluted antibiotics were used to determine the antimicrobial effectiveness of each antibiotic against *S. aureus* and *A. baumannii*.

Results: The incorporation of antibiotics resulted in negligible particle aggregates and porosity within the polymer matrices. Additionally, the residual monomer analysis showed a decrease in conversion of MMA to PMMA due to the addition of antibiotics at 0.5g and 1.0g. The addition of antibiotics also resulted in diverse plasticizing rates as measured by differential scanning calorimetry. The compressive and flexural strength was lower for all of the antibiotics compared to PMMA alone. Elution quantification indicated that the majority of the antibiotics are eluted during Hour 1 and day 1. Antimicrobial effectiveness of the eluent displayed inconsistent results for both 0.5g and 1.0g after Day 2 time point.

Conclusions: Colistin, Polymyxin B, and minocycline were successfully incorporated into PMMA at ratios of 0.5g and 1.0g of antibiotics. The addition of the antibiotics and immersion in water for 30 days did not affect the structural or mechanical properties drastically. Each antibiotic exhibited a burst release profile, except for minocycline at 1.0g which showed a sustained release profile. However, addition of colistin at any concentration and of minocycline at higher concentrations may not be preferred for sustained release because the heterotrophic resistance properties of certain bacterial strains in response to these antibiotics may lead to adverse nosocomial infections.

INTRODUCTION

A common material for fabricating cranial implants is polymethyl methacrylate (PMMA). Antibiotic incorporation into PMMA has been studied for a variety of antibiotics to prevent infection at implant sites [1, 2, 3, 4, 5]. With the rise of infections caused by multi-drug resistant (MDR) pathogens [6, 7], it is of interest to investigate the effects of incorporation of antibiotics effective against these pathogens into PMMA used for cranial implant applications.

Colistin (polymyxin E), polymyxin B, and minocycline are antibiotics effective against MDR gram positive and gram negative bacterial strains, such as methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumanni*. Colistin and polymyxin B, which disrupt bacterial cell membrane function and increases cell permeability, were first introduced as intravenous treatments in the 1950s and later removed from markets in the 1980s, except for treating lung infections in patients with cystic fibrosis [6]. Minocycline, which inhibits bacterial protein sysnthesis, was first introduced in the 1960s as an intravenous treatment and was removed from the US market in 2005 [7]. The polymyxins, mainly colistin, and minocycline were reintroduced as intravenous treatments in the 2000s due to the rise of infections caused by MDR pathogens. Polymyxins have been continuously used for the past few decades as topical otic and ophthalmic treatments.

Literature available for the individual colistin-, polymyxin B-, and minocycline-incorporation into PMMA is limited. In 1976, Chapman and Hadley [8] fabricated colistin- and polymyxin B-incorporated PMMA pellets and observed weak activity against *Escherichia coli*, pencillin-resistant *Staphylococcus aureus*, and pencillin-resistant *Staphylococcus epidermis*. However, more recently, Crane et al. [9] found that colistin-incorporated PMMA beads were more effective than pure PMMA beads and local colistin injections against *Acinetobacter baumanni* over a 7-day period in a mice model. Matos et al. [10] studied the effects of porosity on the release of minocycline from 2.5 w/w% minocycline-incorporated PMMA samples. The authors found that antibiotic released after sample fabrication remained effective against methicillin-susceptible *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecalis*, and *Staphylococcus epidermis*. In these studies, cold-curing PMMA resins were used.

A number of parameters affect antibiotic incorporation into and its elution from PMMA: the brand of PMMA, the curing technique, volume and surface area of the samples,

concentration of antibiotic incorporated, and the intervals between removal and replenishment of surrounding fluid [11, 12]. In this study, colistin, polymyxin B, and minocycline were each added to high-temperature curing PMMA resin at a ratio of 0.5 g and 1.0 g to 40 mL before sample fabrication. Structural analyses and mechanical testing were performed to determine the effects of antibiotic incorporation at different concentrations on the properties of PMMA. Elution kinetics and antimicrobial effectiveness against *S. aureus* and against *A. baumanni* of the eluent collected at different time points were also evaluated.

MATERIALS AND METHODS

Antibiotic incorporation into PMMA

To fabricate each set of antibiotic-incorporated PMMA, the antibiotic was added into Lucite® acrylic resin (Robert B Scott Ocularist, Ltd., Chicago, IL) at a ratio of 0.5 g or 1.0 g of antibiotic to 40 mL of PMMA resin and stirred until homogenously mixed. The dry mixture was slowly added to 12 mL of liquid MMA monomer (Robert B Scott Ocularist, Ltd., Chicago, IL) while stirring continuously. The wet mixture was allowed to sit until dough phase was reached, and was then rolled into the mold shown in Figure 1. The mold was pressed between steel plates and placed into a Hanau curing oven for 8 hours at 71.1 °C. The rectangular rod samples (2 mm width x 2 mm height x 25 mm length) were used for flexural testing and antibiotic elution studies. The cylindrical samples (6 mm diameter x 25 mm length) were used for compression testing. Samples containing 0.5 g and 1.0 g of colistin sulfate (Bioworld, Dublin, OH) were labeled as ‘PMMA_C’ and ‘PMMA_C1’, respectively. Similarly, those containing polymyxin B sulfate (Alfa Aesar, Ward Hill, MA) were labeled ‘PMMA_PB’ and ‘PMMA_PB1’, and those containing minocycline hydrochloride (Sigma Aldrich, St. Louis, MO) were labeled ‘PMMA_M’ and ‘PMMA_M1’. Pure PMMA samples were also fabricated and labeled as ‘PMMA’.

Molecular weight analysis

Gas permeation chromatography (GPC) was performed to determine the average molecular weights of each PMMA set. Samples from each set were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at 10 mg/mL and passed through 0.45 µm Teflon filters. A volume of 25 µL of each dissolved and filtered sample was injected at a flow rate of 1.0 mL/min into a Waters Alliance 2695 separations module equipped with a Agilent PL HFIP gel column

(300 x 7.5 mm, 40 ºC) and a differential refractive index detector (128x attenuation, 40 ºC). Calibration was performed for a molecular weight range of 550–2,136,000 g/mol using PMMA standards prepared by dissolving a standard mixture from an EasiVial PMMA kit (Agilent, Santa Clara, CA). Analysis of each PMMA sample set was performed in triplicate.

Residual monomer analysis

A piece of each sample (118.2 mg of PMMA, 109.4 mg of PMMA_C, 137.2 mg of PMMA_C1, 114.5 mg of PMMA_PB, 104.6 mg of PMMA_PB1, 123.1 mg of PMMA_M, and 106.6 mg of PMMA_M1) was placed individually in a 25 mL volumetric flask. A volume of 3 mL of chloroform was added to each flask and the flasks were placed in a sonic bath for 60 minutes to completely dissolve the polymer samples. The flasks were then filled to volume with methanol to precipitate out the polymer. The liquid in each flask was passed through a 0.45 µm Teflon syringe filter directly into an auto-sampler vial.

For the calibration solutions, a stock solution of MMA (Sigma Aldrich, St. Louis, MO) was prepared by weighing out 55.6 mg of MMA into a 25 mL volumetric flask. Methanol was added to fill the flasks to volume to dissolve the MMA. The stock solution was further diluted to obtain solutions with concentrations of 0.1112, 0.2224, 0.4448, and 0.6672 mg/mL MMA in methanol.

Sample, calibration, and blank solutions were analyzed using a Phenomenex ZB-5MS (30 m x 0.25 mm x 1.0 µm) equipped with a flame ionization detector set to 300 °C. The inlet temperature was 225 °C and the transfer line temperature was 300 °C. A split injection of 1 µL was used with a split ratio of 20:1. The temperature program used was a 3 minute hold at 45 °C, a 20 °C/min increase to 280 °C, and a 2 minute hold at 280 °C. The column had a carrier gas (He) flow rate of 1 mL/min, and the detector had a H_2 gas flow rate of 35 mL/min, air flow rate of 350 mL/min, and a make-up gas flow rate of 20 mL/min. Analysis of each PMMA sample set was performed in triplicate.

Differential scanning calorimetry

The glass transition temperature (T_g) of each PMMA material was determined using differential scanning calorimetry (DSC) according to ASTM E1356-08: Standard Test Method for Assignment of the Glass Transition Temperatures by Differential Scanning Calorimetry. The

DSC system used was a Mettler Toledo DSC823^{e} with STARe analysis software (Metter Toledo, Columbus, OH). A mass of 5 mg from each material was weighed into an aluminum pan and subjected to the following heating program alongside an empty reference pan: heating from 25 °C to 160 °C at 10 °C/min, holding at 160 °C for 3 minutes, cooling from 160 °C to 25 °C at 20 °C/min, holding at 25 °C for 3 minutes, heating at 25 °C to 225 °C at 10 °C/min, and cooling from 225 °C to 25 °C at 20 °C/min. Per ASTM E1356-08, the value of T_g for each material was determined using data from the second heating period to evaluate how each antibiotic affected the inherent property of the material. The first heating period (above the melt temperature of PMMA) followed by the controlled cooling period erased the original thermal history of the samples and provided the samples with a known thermal history for comparison.

PMMA immersion for antibiotic elution

Five rectangular rods from each sample set were immersed individually in MilliQ water with constant shaking for up to 30 days. Eluent was removed and replenished at different time points: hour (Hr) 1, Day 1, Day 2, Day 3, Day 4, Day 7, Day 15, Day 22, and Day 30. The eluent removed at each time point was stored at -80 °C for further analysis.

Compression testing

Compression testing was carried out with reference to ISO 5833: Implants for surgery — Acrylic resin cements. To meet the recommendations of the standard, the mold samples were cut in half and then sized to cylindrical samples that were 6 mm in diameter and 12 mm in length. Compression testing was carried out using an Instron ElectroPuls^{TM} E3000 with Bluehill® 3 software (Instron, Norwood, MA) at a 20 mm/min constant cross-head speed. Five samples from each PMMA set were tested after fabrication to determine the compressive strength of each antibiotic-incorporated PMMA compared to that of control PMMA. Five cylindrical rod samples from each set were also immersed in water for 30 days and tested to determine the effects of immersion on the average compressive strength of each set.

Flexural testing

Three-point bend testing was carried out with reference to ISO 4049: Dentistry — Polymer-based restorative materials. ISO 5083 was not used for flexural testing because of the

limitation in the size of the curing oven and sample mold available for the study. Tests were carried out using an Instron ElectroPuls^{TM} E3000 with Bluehill® 3 software (Instron, Norwood, MA) at a 1 mm/min constant cross-head speed. Five samples from each PMMA set were tested after fabrication to determine the flexural strength and flexural modulus of each antibiotic-incorporated PMMA compared to those of control PMMA. The five samples from each set that were immersed in water for 30 days were also tested to determine the effects of immersion on average flexural strength and average flexural modulus of each sample set.

Elution quantification

Quantification of colistin and polymyxin B in eluent obtained at different time points from PMMC_C, PMMA_C1, PMMA_PB, and PMMA_PB1 was performed using HPLC with UV detection (HPLC/UV), while quantification of minocycline in eluent obtained at different time points from PMMA_M and PMMA_M1 were performed using HPLC with tandem mass spectrometry (HPLC/MS/MS).

Colistin

The HPLC/UV system consisted of two Shimadzu LC-20AD pumps, a Shimadzu CTO-20A column oven, and a Shimadzo SOD-20A dual wavelength absorbance detector at $\lambda = 214$ nm (Kyoto, Japan) coupled with an ACE Excel C18 PFP analytical column (Aberdeen, Scotland), maintained at 40 °C during the analysis. The mobile phases were run at 0.5 mL/min. Mobile phase A was 4.5% HPLC grade acetonitrile (Fisher Scientific, Fairlawn, NJ) and 95.5% 12 mM KH_2PO_4 at pH 2.5. Mobile phase B was 85% acetonitrile and 15% 12 mM potassium phosphate monobasic (Fisher Scientific, Fairlawn, NJ) at pH 2.5. During each run, the binary gradient consisted of mobile phase A for 0–1 minutes, mobile phase B for 1–12 minutes, and mobile phase A for 12.1–16 minutes.

A colistin working solution containing 100 μg of colistin sulfate (USP, Rockville, MD) per 1 mL of MilliQ water was used to spike the calibrator solution, and a bupivicaine working solution containing 100 μg of bupivicaine hydrochloride (Sigma Aldrich, St. Louis, MO) per 1 mL of MilliQ water was used as an internal standard. Calibrator solution and each eluent sample were mixed separately with bupivacaine working solution at volume ratio of 500 μL to 50 μL and vortexed for 1 minute, transferred to glass culture tubes, and dried to powder form using a

steady stream of nitrogen gas. The dried samples were re-suspended in 50 µL of methanol (Fisher Scientific, Fairlawn, NJ) and 20 µL were injected into the HPLC system. A linear regression calibration curve was created using calibrator response ratios of 0, 5, 10, 20, and 40 µg/mL colisitin. The ratio of the peak area of colistin to that of the bupivicaine standard was compared to the calibration curve to determine the quantity of colistin in each eluent sample. The detection limit for the quantification of colistin was 2.5 µg/mL.

Polymyxin B

The procedure for quantification of colistin in PMMA_C and PMMA_C1 eluent samples was repeated for quantification of polymyxin B in PMMA_PB and PMMA_PB1 eluent samples, with the replacement of colistin with polymyxin B to produce the calibration curve.

Minocycline

The HPLC/MS/MS system consisted of two Shimadzu SCL-10A controllers, a Shimadzu LC-10AD with an FCV-10AL mixing chamber, Shimadzo SIL-10AD auto-sampler (Kyoto, Japan), and an AB Sciex API 3200 tandem mass spectrometer with turbo ion spray (Framingham, MA) coupled with an ACE Excel C18 PFP analytical column (Aberdeen, Scotland), maintained at 60 °C during the analysis. The mobile phases were run at 0.4 mL/min at a mobile phase A to mobile phase B ratio of 70 to 30. Mobile phase A was 99.9% MilliQ water and 0.1% formic acid (Sigma Aldrich, St. Louis, MO). Mobile phase B was 99.9% acetonitrile and 0.1% formic acid.

A minocycline working solution containing 100 µg of minocycline hydrochloride (Sigma Aldrich, St. Louis, MO) per 1 mL of MilliQ water was used to spike the calibrator solution, and a tetracycline working solution containing 10 µg of tetracycline hydrochloride (Sigma Aldrich, St. Louis, MO) per 1 mL of MilliQ water was used as an internal standard. A volume of 100 µL of calibrator solution or 100 µL of each eluent sample were mixed with 10 µL of tetracycline working solution and vortexed for 30 seconds, transferred to auto-sampler vials, and injected into the HPLC system. A linear regression calibration curve was created using calibrator response ratios of 0, 0.05, 0.1, 1, 5, 10, 25, and 50 µg/mL minocycline. The ratio of the peak area of minocycline to that of the tetracycline standard was compared to the calibration curve to

determine the quantity of minocycline in each eluent sample. The detection limit for quantification of minocycline was 0.05 µg/mL.

Antimicrobial effectiveness

Two-fold serial dilution minimum inhibitory concentration (MIC) assays were performed for each antibiotic from 512 µg/mL to 0.25 µg/mL against gram positive methicillin resistant *Staphylococcus aureus* subsp. *aureus* (ATCC® 33591™, referred to as *S. aureus* for the remainder of the paper) and gram negative multidrug resistant *Acenitobacter baumanii* (ATCC® BAA-747™, referred to as *A. baumanii* for the remainder of the paper), which were obtained from ATCC (Manassas, VA). Eluent collected from each antibiotic incorporated rod sample at each time point was assayed on the same 96-well plate as the serial dilution of the corresponding antibiotic to determine whether the amount of antibiotic eluted from each rod at each time point was above or below MIC for that assay. Bacterial strains were cultured and the MIC evaluation assays were run per ATCC recommendations. Assays for *A. baumanii* and *S. aureus* were run overnight at 37 °C with a bacterial concentration of 5×10^5 colony forming units (CFUs) in tryptic soy broth and in nutrient media broth (Sigma Aldrich, St. Louis, MO), respectively. For consistency with the sample eluent wells in each assay, the serial dilution wells, positive control wells containing only bacteria, and negative control wells containing no antibiotic or bacteria were prepared such that the final volume of liquid in each well consisted of 100 µL of MilliQ water and 100 µL of media. Plates were analyzed in a BioTek Synergy HT microplate reader (Winooski, VT) at OD600 to evaluate growth or inhibition of growth in each well.

Statistical Analysis

Statistical analyses were performed by one-way ANOVA analyses and Dunnet's multiple comparisons tests ($p < 0.05$, CI 95%) or unpaired t-tests with Welch's correction ($p < 0.05$, CI 95%) using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Particle aggregates (bright areas in Figure 2) were observed in the polymer matrix (dull areas in Figure 2) in PMMA_C and large pores (dark areas in Figure 3) were present in

PMMA_M1. The remaining antibiotic-incorporated PMMA materials had negligible particle aggregates and porosity within their polymer matrices.

Molecular weight analysis

The number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity ($D = M_w/M_n$), which represents the molecular weight distribution, determined for each sample set using GPC are listed in Table 1. Addition of 0.5 g of antibiotic resulted in statistically significant decreases in M_n for PMMA_M and M_w for PMMA_C and PMMA_M as well as a statistically significant increase in D for PMMA_M compared to those for PMMA were found. Addition of 1.0 g of antibiotic resulted in statistically significant increases in M_n and M_w as well as statistically significant decreases in D for PMMA_C1, PMMA_PB1, and PMMA_M1 compared to those for PMMA were found.

Residual monomer analysis

Residual monomer analysis was performed to determine if the presence of antibiotic interfered with polymerization and increased the quantities of unreacted MMA present in the cured samples. Results showed that all three antibiotic-incorporated PMMA samples had statistically higher MMA contents compared to that in the control PMMA (Table 1).

The residual monomer content was used to determine the degree of conversion of MMA to PMMA using the formula $X = 1 - W^*/100$, where W* is the weight fraction of the residual monomer based on the initial weight of monomer used [13]. The decrease in degree of conversion of MMA to PMMA due to the addition of each antibiotic at 0.5 g was statistically significant. Addition of 1.0 g of antibiotic resulted in a significantly greater decrease in MMA conversion (Table 1).

Differential scanning calorimetry

Antibiotic incorporation into PMMA has been found to have a plasticizing effect, which can result in decreases in mechanical properties. The values of T_g listed for each material in Table 1 show that incorporation of 0.5 g of colistin and 1.0 g of polymyxin B resulted in an increase in T_g of PMMA, while the incorporation of 1.0 g of colistin, 0.5 g of polymyxin B, and

0.5 g of minocycline resulted in decreases in T_g of PMMA. Incorporation of 1.0 g of minocycline resulted in no observable change in T_g.

Compression testing

The average compressive strength of each material is listed in Table 2. The average compressive strength of PMMA_PB was statistically similar to that of PMMA, while the average compressive strengths of the remaining antibiotic-incorporated PMMA materials were each statistically lower than that of PMMA. The average compressive strengths of PMMA_PB1 and PMMA_M1 were statistically lower than those of PMMA_PB and PMMA_M, respectively, while the average compressive strength of PMMA_C1 was statistically similar to that of PMMA_C. The reduction in average compressive strengths of PMMA and all antibiotic-incorporated PMMA except PMMA_C changed significantly due to immersion in water for 30 days. In general, however, after fabrication and after immersion all antibiotic incorporated samples had compressive strengths higher than the ISO 5833 recommended value of 70 MPa (Table 2).

Flexural testing

The average flexural strength and average flexural modulus of each material is listed in Table 2. The average flexural strengths of PMMA_PB and PMMA_M were statistically similar to that of PMMA, while the average flexural strengths of the remaining antibiotic-incorporated PMMA were each statistically lower than that of PMMA. The average flexural strength of PMMA_C after immersion was statistically lower than that before immersion; while the average flexural strengths of PMMA and the remaining antibiotic-incorporated PMMA were each statistically similar to their respective average flexural strengths before immersion.

Addition of 0.5 g of antibiotic did not result in a statistically significant change in average flexural moduli of PMMA, while addition of 1.0 g of antibiotic did. The average flexural moduli of PMMA_PB1 and PMMA_M1 were statistically lower than those of PMMA_PB and PMMA_M, while the average flexural modulus of PMMA_C1 was not statistically different to that of PMMA_C. The average flexural moduli of PMMA_C and PMMA_PB1 after immersion were statistically lower due to immersion for 30 days, while the average flexural moduli of

PMMA and the remaining antibiotic-incorporated PMMA did not undergo statistically significant changes due to immersion.

Elution quantification

A burst release profile was observed for each antibiotic incorporated PMMA material, as seen in Figures 4, 5 and 6. Based on the detection limit of 0.25 µg/mL for colistin and polymyxin B, lines labeled PMMA_X (where X is C, C1, PB or PB1) and lines labeled PMMA_X_0 (where X is C, C1, PB or PB1) were drawn in Figures 4 and 5 to indicate the maximum and minimum of antibiotic that eluted at each time point during the 30 day period. The majority of antibiotic eluted from PMMA_C, PMMA_C1, PMMA_M, and PMMA_M1 material during Hr 1 and Day 1, and from PMMA_PB and PMMA_PB1 during Hr 1, indicating that elution took place primarily from sample surfaces during the 30 day period. Taking the detection limits into account, PMMA_M1 was the only material that exhibited (detectable) sustained release of antibiotic during the 30 day period.

Antimicrobial effectiveness of eluents

The results for antimicrobial effectiveness of each antibiotic eluent (Table 3) and the MIC for the serial dilution of each antibiotic against *S. aureus* and *A. baumanni* are listed in Table 3. Eluent obtained from PMMA_C and from PMMA_C1 M1 inhibited growth of *A. baumanni* inconsistently after the Day 2 time point and PMMA_M1 inhibited growth of *S. aureus* inconsistently after the Day 2 time point.

DISCUSSION

Structural characteristics and mechanical properties were studied for each antibiotic-incorporated PMMA and compared to those of control PMMA. PMMA_C had large regions of antibiotic particle aggregates (Figure 2), indicating that the antibiotic was not homogenously dispersed throughout the polymer matrix. The porosity observed in PMMA_M1 formed within the material during the fabrication process. However, it is also possible that during cutting and polishing to prepare the surface for imaging antibiotic aggregates may have become dislodged resulting in an apparent porosity. The absence of antibiotic aggregates in the remaining

antibiotic-incorporated PMMA indicated that the antibiotic was homogenously dispersed within their polymer matrices.

Changes in molecular weights observed due to antibiotic-incorporation may have been due to antibiotic molecules acting like chain transfer agents, the presence and concentrations of which affected polymer chain growth during polymerization. Increases in residual MMA content corresponding to decreases in MMA conversion in all antibiotic-incorporated PMMA samples was likely due to physical, electrostatic, and chemical interactions between antibiotic and monomer molecules during polymerization [14].

Changes in T_g are indicative of changes in structure that occur in the material and affect the mechanical properties of the material. Various properties affect the T_g of a polymer. Increases in the concentrations of residual MMA and antibiotics, which act as plasticizers [13, 15], decrease T_g; increases in Mw [16, 17], D [17], and porosity [18]increase T_g. In light of the properties of PMMA listed in Table 1, the following explanations for the T_g of each antibiotic-incorporated PMMA can be made. PMMA_C had a higher T_g compared to that of PMMA because of the presence of aggregates and their associated porosity that counteracted the effects of the higher MMA content and lower M_w of PMMA_C. PMMA_C1 had a lower T_g compared to that of PMMA because the higher MMA content and lower D counteracted the effects of the higher M_w of PMMA_C1. PMMA_PB had a lower T_g compared to that of PMMA because the higher MMA content and lower M_w counteracted the effects of the higher D of PMMA_PB. PMMA_PB1 had a higher T_g compared to that of PMMA because of higher M_w counteracted the effects of the higher MMA content and lower D of PMMA_PB1. This observation for PMMA_PB1 was opposite to that for PMMA_C1 and PMMA_PB. Another explanation for the increase in T_g for PMMA_PB1 may be that the greater number of polymyxin B molecules interacting with the polymer chain produced a crosslinking effect [19], resulting in an apparent higher M_w of the polymer molecules and increasing T_g. PMMA_M had a lower T_g compared to that of PMMA because the higher MMA content and lower M_w counteracted the effects of the higher D of PMMA_M. PMMA_M1 had a T_g similar to that of PMMA because the increase in MMA content and decrease in D nullified the effects of the increase in Mw and the presence of porosity.

Greater decreases in M_w and greater amounts of residual MMA in PMMA_C and PMMA_M compared to PMMA_PB likely resulted in the significantly lower compressive

strengths of PMMA_C and PMMA_M compared to PMMA. Although Mw significantly increased for the 1.0 g antibiotic-incorporated PMMA materials, the greater amounts of MMA and antibiotic present in each material resulted in significant decreases in compressive strength compared to their 0.5 g counterparts.

The significant decrease in flexural strength observed for PMMA_C compared to those for PMMA_PB and PMMA_M can be explained by the presence of aggregates and their corresponding porosity present in PMMA_C, which act as stress concentrators in the lower half of the sample that is in tension [20, 21]. Similar to the compressive strength, the flexural strength of the 1.0 g antibiotic-incorporated PMMA materials decreased significantly due to the greater amounts of MMA and antibiotic present in these samples compared to their 0.5 g counterparts. In addition, the significant decrease in flexural moduli of PMMA after incorporation of 1.0 g of antibiotic was related to the significant increases in M_n and M_w.

During immersion, three parallel processes occur that cause aging of the polymer matrix by inducing changes in polymer structural and materials properties: natural aging due to continued polymerization of residual monomer within the polymer matrix; adsorption of water into the polymer matrix; and diffusion of residual monomer and antibiotic, if present, from the material [22]. The time period of immersion and the rate at which these processes occur also affect the stage of the ageing process. The difference between the values of the compressive and flexural strengths and the flexural moduli before and after immersion for each material varied. The stage of the PMMA matrix aging process that each material was in at the 30 day time period likely contributed to their different properties after immersion.

During immersion, elution of molecules from the PMMA matrix is believed to start with burst release at the surface, followed by dissolution within water that has been absorbed by the matrix and diffusion through the matrix to the surface [23]. Elution is affected by physical characteristics of the eluting molecules such as molecular weight (heavier molecules move slower than lighter molecules), molar concentration (a higher concentration increases the gradient for diffusion) [12], and chemical characteristics such as molecular interactions (between the diffusing molecules and the diffusion medium) [19].

Minocycline has a lower molecular weight (493.9 g/mol) than that of colistin (1267.6 g/mol) and polymyxin B (1385.63 g/mol). As such, the molar quantity of minocycline added was 2.56 times that of colistin and 2.8 times that of polymyxin B, which resulted in minocycline

(Figure 6) eluting at higher quantities than colistin (Figure 4) and polymyxin B (Figure 5) did. Considering the eluted amounts during the first 24 hours (Hr 1 and Day 1 time points), the molar quantity of minocycline that eluted from PMMA_M was 4.16 times that from PMMA_C and 3.61 times that from PMMA_PB, and the molar quantity of minocycline that eluted from PMMA_M1 was 8.87 times that from PMMA_C1 and 9.54 times that from PMMA_PB1. Doubling the quantity of each antibiotic added to PMMA resulted in an increase in elution of colistin by 2.49 times, polymyxin B by 2.01 times, and minocycline by 5.30 times. Higher molar quantities of each antibiotic incorporated into PMMA increased the quantity of antibiotic on the surfaces of each sample and increased the diffusion gradient between the sample surfaces and the surrounding media. The increase in antibiotic quantity and increase in diffusion gradient both led to the increase in the amount of antibiotic burst released from surfaces during the first 24 hours of immersion [11].

Bacterial studies provided evidence that antibiotics released from PMMA had retained their antimicrobial effectiveness after undergoing the high-temperature polymer curing process. Correlation was observed between the antibiotic quantities measured in the eluents and the MIC values for *S. aureus* assays for each sample. The measured colistin (< 2.5 μg), polymyxin B (< 2.5 μg), and minocycline (≤ 0.698 μg) quantities in the eluents of PMMA_C, PMMA_PB, and PMMA_M, respectively, from Day 2 to Day 30 were below the respective MIC range (Table 3). In the case of PMMA_PB, it was observed that the Day 1 eluent had inhibited bacterial growth. It is possible that the quantity of antibiotic in the eluent was between 2 μg/mL (the minimum value of the MIC range) and 2.5 μg/mL (the detection limit of the quantitation process). The measured colistin (≤ 4.294 μg) and polymyxin B (< 2.5 μg) quantities in the eluents of PMMA_C1 and PMMA_PB1, respectively, from Day 1 to Day 30 were below the respective MIC value (Table 3). In the case of PMMA_M1, inconsistent results were obtained for the measured minocycline quantities (≤ 1.24 μg) from Day 2 to Day 30.

Correlation was also observed between the antibiotic quantities measured in the eluents and the MIC values for *A. baumanni* for PMMA_PB, PMMA_PB1, PMMA_M, and PMMA_M1. The measured polymyxin B quantities (< 2.5 μg/mL) in the PMMA_PB eluent from Day 2 to Day 30 were below MIC, and the measured minocycline quantities (< 0.05 μg/mL) in the PMMA_M eluent from Day 7 to Day 30 were below MIC. In addition, the measured polymyxin B (< 2.5 μg/mL) and minocycline (< 0.06 μg/mL) quantities in the eluents

of PMMA_PB1 and PMMA_M1, respectively, for Day 30 were below the respective MIC values (Table 3).

The inconsistent results for the antibacterial effectiveness of the PMMA_C and PMMA_C1 eluents taken at time points after Day 2 and Day 1, respectively, may be explained as follows. On one plate for each rod, eluents at later time points were observed to inhibit growth, while eluents from earlier time points were not observed to inhibit growth (for example, growth occurred for eluent from Day 15 and Day 22, but was inhibited for eluent from Day 30). Because colistin particles formed aggregates that were inhomogeneously dispersed within the PMMA matrix, it is possible that at certain time points colistin molecules from these aggregates diffused to the surface of the material and were released, thus resulting in inconsistent inhibition of growth by the eluent of each rod at later time points. Another explanation of the inconsistency of inhibition of growth not just between later times points on each plate, but between plates as well, is the heteroresistance property of *A. baumanni* against colistin. In 2006, Li et al. [24]published findings that *A. baumanni* exposed to colistin at moderate levels above the MIC level exhibited heteroresistance such that certain subpopulations became resistant to colistin during testing. In the current study, the MIC range was found to be 0.5–4 μg/mL. It is possible that the eluent from certain time points contained quantities of colistin that were greater than MIC (but lower than the quantification limit of 2.5 μg/mL) and in a range that resulted in the heteroresistance of *A. baumanni* coming into effect. Heteroresistance has been found to occur for numerous bacterial strain-antibiotic pairs, and should be taken into consideration when determining which antibiotic to use in drug eluting implant applications [25]. It is possible that a similar phenomenon occurred during the assays for the eluent from PMMA_M1 against *S. aureus* leading to the inconsistent results after the Day 1 time point. Further investigation into the effectiveness of minocycline against *S. aureus* strains when minocycline is present at moderate levels above the MIC should be carried out.

In conclusion, colistin, polymyxin B, and minocycline were successfully incorporated into PMMA at ratios of 0.5 g and 1.0 g of antibiotic to 40 mL of resin and each antibiotic eluted from PMMA. Addition of the antibiotics and immersion in water for 30 days did not drastically affect structural or mechanical properties of PMMA to render the material unserviceable. Each antibiotic exhibited burst release profiles from PMMA at 0.5 g and 1.0 g incorporation. In addition, minocycline exhibited a (detectable) sustained release profile when incorporated at 1.0

g. Bacterial studies provided evidence that each antibiotic remained effective against *S. aureus* and *A. baumanni* even after the high-temperature curing process. However, addition of colistin at any concentration and of minocycline at higher concentrations may not be preferred for sustained release because the heteroresistance properties of certain bacterial strains in response to these antibiotics may lead to adverse nosocomial infections. Future studies to improve sustained release of polymyxin B will include addition of soluble fillers to enhance elution of polymyxin B from PMMA.

MILITARY SIGNIFICANCE

The current study demonstrated the successful incorporation of antibiotics into PMMA to treat cranio-maxillofacial defects for service members injured in combat operations. Although the bactericidal activity of the antibiotics was inconsistent, the data demonstrated that the antibiotics retained effectiveness after exposure to the high temperature needed to cure PMMA. Additionally, the structural and mechanical analysis of the antibiotic incorporated PMMA rods were not drastically changed. Future studies to improve sustained release of polymyxin B will include addition of soluble fillers to enhance elution of polymyxin B from PMMA.

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Table 1. Average and standard deviation values for M_n, M_w, D, residual MMA, X, and T_g of PMMA and antibiotic-incorporated PMMA samples.

	M_n (g/mol)	M_w (g/mol)	D	MMA (%)	X	T_g (°C)[c]
PMMA	140393 ± 1487	390292 ± 2857	2.782 ± 0.026	5.6615 ± 0.0083	0.7056 ± 0.0004	105.0
PMMA_C [b]	137036 ± 7630	378315 ± 1920[a]	2.766 ± 0.136	6.6043 ± 0.014[a]	0.6566 ± 0.0005[a]	106.5
PMMA_C1	185348 ± 16377[a]	421866 ± 10836[a]	2.285 ± 0.1664[a]	7.173 ± 0.07234[a]	0.6207 ± 0.0038[a]	104.5
PMMA_PB [b]	134817 ± 2725	387199 ± 1977	2.873 ± 0.062	6.3227 ± 0.0485[a]	0.6713 ± 0.0025[a]	103.8
PMMA_PB1	215537 ± 4491[a]	444881 ± 2894[a]	2.064 ± 0.0295[a]	6.97 ± 0.06557[a]	0.6314 ± 0.0035[a]	106.0
PMMA_M [b]	124785 ± 4190[a]	378101 ± 1639[a]	3.032 ± 0.090[a]	7.2857 ± 0.0297[a]	0.6212 ±0.001[a]	104.6
PMMA_M1	197217 ± 7547[a]	437352 ± 5124[a]	2.219 ± 0.0604[a]	8.63 ± 0.07937[a]	0.5437 ±0.0042[a]	105.0

[a] indicates statistically significant difference with PMMA data ($p < 0.05$)
[b] indicates statistically significant difference between the value of each parameter with that of the corresponding 1.0 g AB sample
[c] per ASTM 1356, no standard deviation for Tg was obtained

Table 2. Average compressive strength, flexural modulus, and flexural strength of each sample set before and after immersion.

Material	Compressive Strength (MPa)		Flexural Strength (MPa)		Flexural Modulus (MPa)	
	Before	**After**	**Before**	**After**	**Before**	**After**
PMMA	130.4 ± 1.9	119.1 ± 1.6[c]	94.2 ± 3.7	96.1 ± 17.1	2438 ± 161	2694 ± 806
PMMA_C	117.8 ± 4.7[a]	113.1 ± 2.8[a]	79.5 ± 10.8[a,b]	66.3 ± 4.0[a,c]	2400 ± 154	1949 ± 100[a,c]
PMMA_C1	121.8 ± 1.6[a]	101.2 ± 2.6[a,c]	68.4 ± 5.6[a]	69.7 ± 7.5	2086 ± 325[a]	2195 ± 175
PMMA_PB	126.1 ± 2.0[b]	113.4 ± 2.8[a,c]	83.6 ± 2.9[b]	77.1 ± 7.7[a]	2448 ± 126[b]	2320 ± 139
PMMA_PB1	118.3 ± 2.9[a]	101.9 ± 0.97[a,c]	64.57 ± 11.3[a]	64.5 ± 4.2	2055 ± 103[a]	2266 ± 93.5[c]
PMMA_M	119.5 ± 1.8[a,b]	112.5 ± 1.7[a,c]	84.2 ± 10.3[b]	79.6 ± 2.4[a]	2600 ± 488[b]	2136 ± 206
PMMA_M1	114.2 ± 2.4[a]	103.2 ± 2.4[a,c]	60.1 ± 5.3[a]	75.1 ± 15.9	1754 ± 99.5[a]	2283 ± 690

[a] indicates statistically significant difference with PMMA data ($p < 0.05$)
[b] indicates statistically significant difference with corresponding 1.0 g antibiotic PMMA sample data ($p < 0.05$)
[c] indicates statistically significant difference with before immersion data ($p < 0.05$)

Table 3. List of elution time points at which eluent from samples was no longer effective against *S. aureus* and *A. baumanni* and the MIC values obtained for each antibiotic for the corresponding assay. The MIC range is given as a range in which growth was observed at the minimum concentration and no growth was observed at the maximum concentration.

	S. aureus	***A. baumanni***

Material	Elution time point	MIC (µg/mL)	Elution time point	MIC (µg/mL)
PMMA_C	Day 2	4–8	Inconsistent after Day 2*	0.5–4
PMMA_C1	Day 1	32–64	Inconsistent after Day 1*	0.25–1
PMMA_PB	Day 2	2–4	Day 2	0.5–2
PMMA_PB1	Day 1	32–64	Day 30	<4
PMMA_M	Day 2	4–8	Day 7	<0.25
PMMA_M1	Inconsistent after Day 2*	0.5–2	Day 30	<1

*Growth varied in wells for the same time point eluent among the experimental triplicates

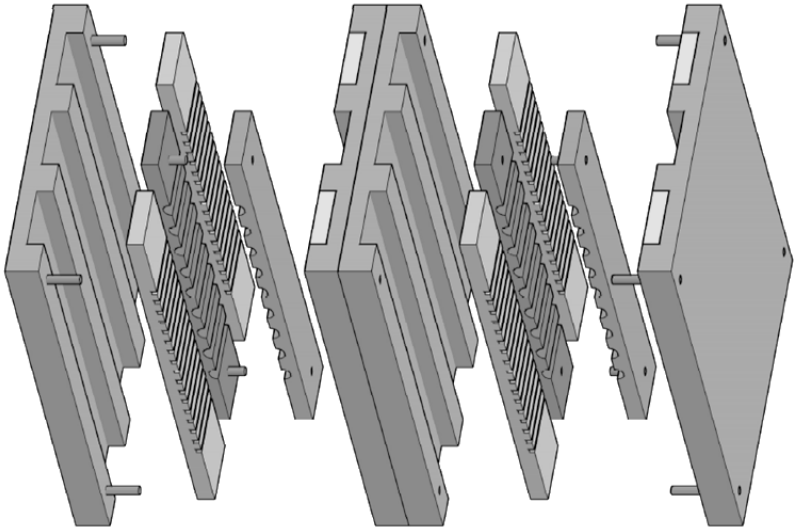

Figure 1. The Teflon® mold used to produce the antibiotic-incorporated PMMA sample sets. Rectangular rod samples for flexural testing and antibiotic elution studies were fabricated in the side rows. Cylindrical samples for compression testing were fabricated in the middle rows.

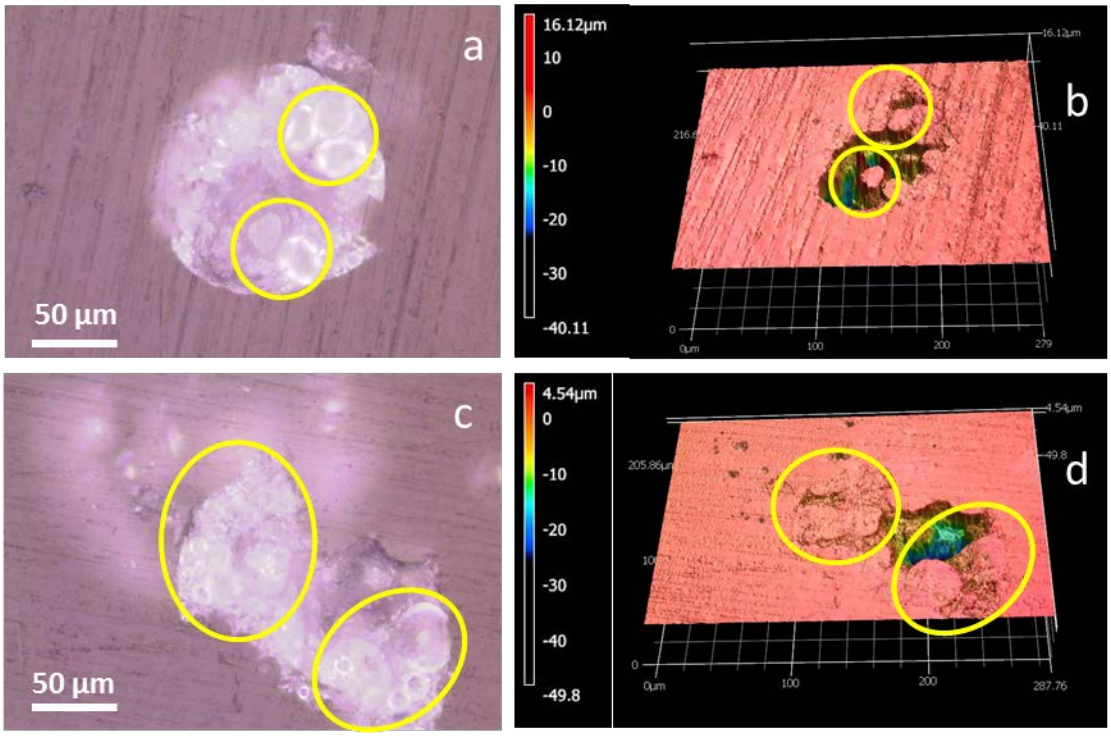

Figure 2. Confocal laser scanning microscope (CLSM) images of colistin particle aggregates found in PMMA_C. Yellow circles in the optical images (a) and (c) and the 3D CLSM images (b) and (d) of the same regions, respectively, indicate corresponding regions of antibiotic aggregates within the material.

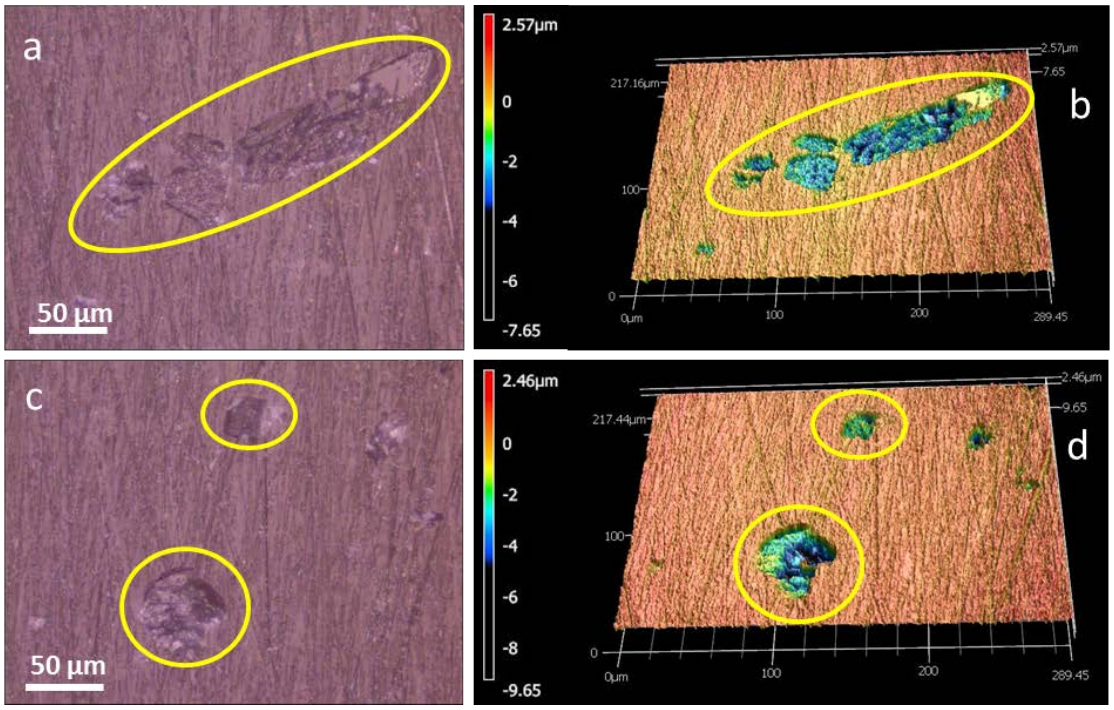

Figure 3. Confocal laser scanning microscope (CLSM) images of pores found in the PMMA_M1 samples. Yellow circles in the optical images (a) and (c) and the 3D CLSM images (b) and (d) of the same regions, respectively, indicate corresponding pores within the material.

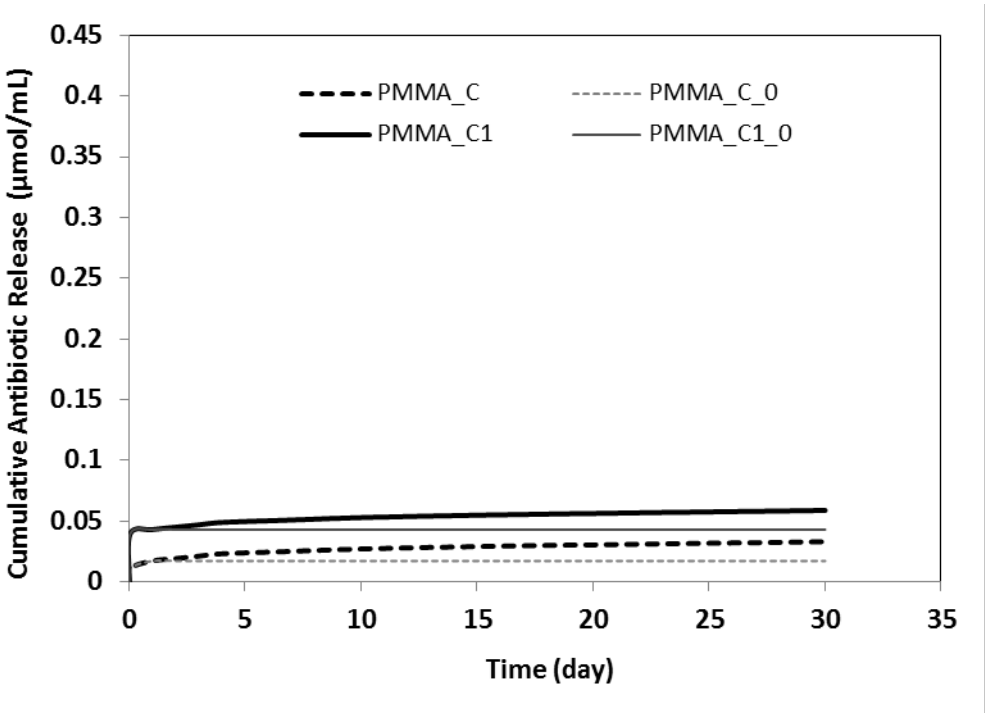

Figure 4. Cumulative molar quantity of colistin eluted from PMMA_C and PMMA_C1 at baseline (PMMA_C_0, PMMA_C1_0) and each time point. The quantity of colistin eluted from both materials after the Day 1 time point was less than the detection limit of 0.0020 µmol/mL (2.5 µg/mL). For later time points, the quantity of colistin released fell between PMMA_C and PMMA_C_0 and between PMMA_C1 and PMMA_C1_0.

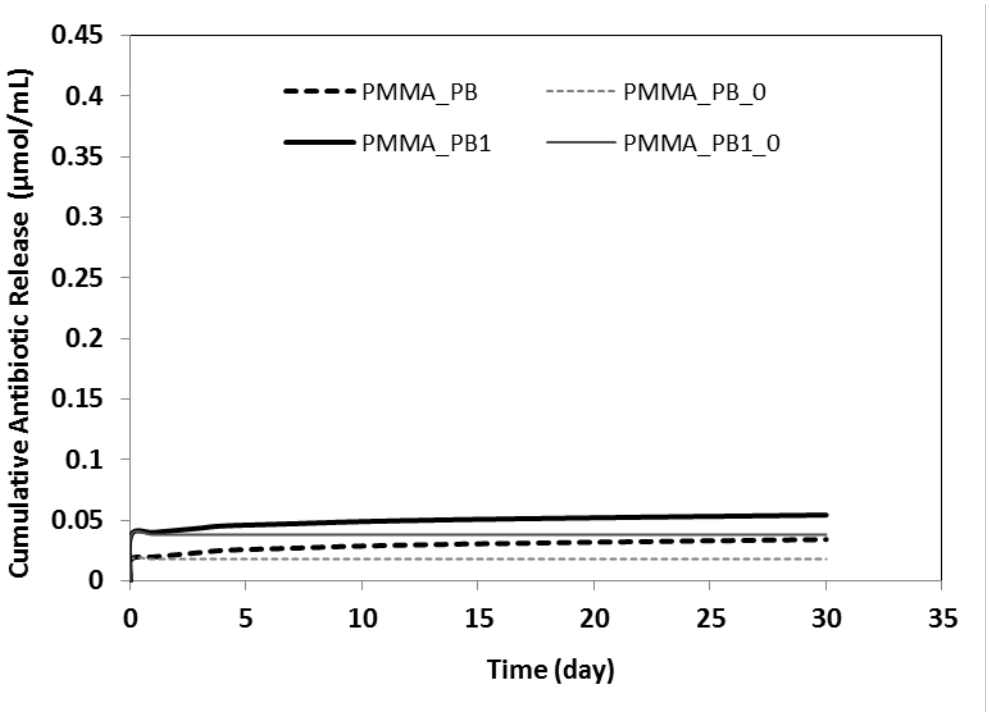

Figure 5. Cumulative molar quantity of polymyxin B eluted from PMMA_PB and PMMA_PB1 at baseline (PMMA_PBC_0, PMMA_PBC1_0) and each time point. The quantity of polymyxin B eluted from both materials after the Hr 1 time point was less than the detection limit of 0.0018 µmol/mL (2.5 µg/mL) after the Hr 1 time point. For later time points, the quantity of polymyxin B released fell between PMMA_PB and PMMA_PB_0 and between PMMA_PB1 and PMMA_PB1_0.

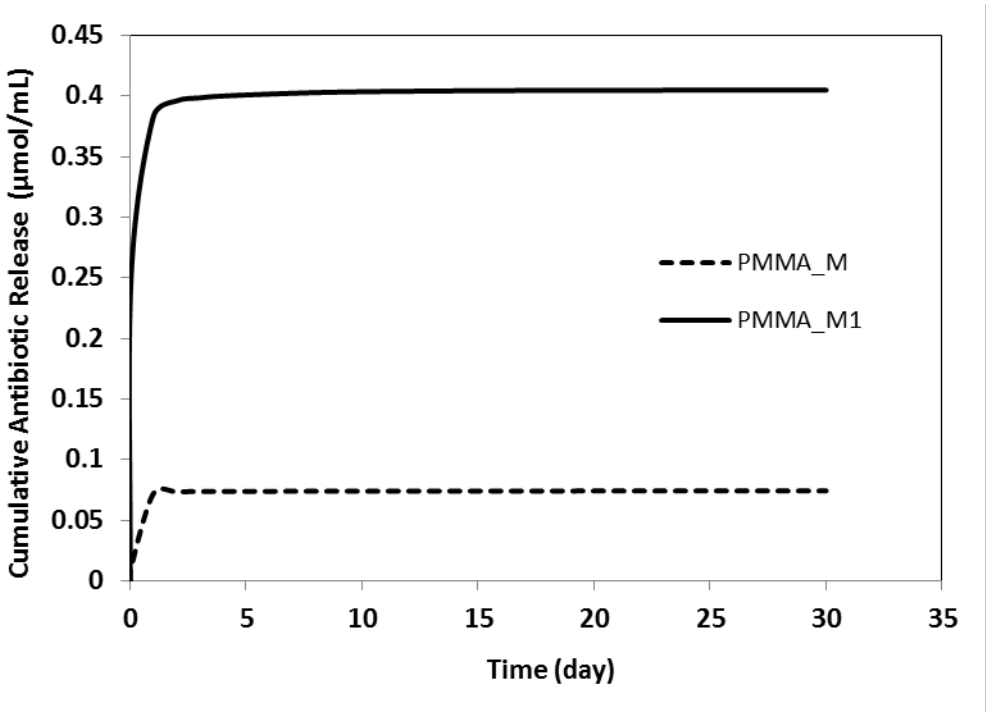

Figure 6. Cumulative molar quantity of minocycline eluted from PMMA_M and PMMA_M1 at each time point. The quantity of minocycline eluted from PMMA_M after the Day 3 time point and the quantity of minocycline eluted from PMMA_M1 after the Day 22 time point was less than the detection limit of 0.001 µmol/mL (0.05 µg/mL).

REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD MM YY)	2. REPORT TYPE	3. DATES COVERED (from – to)
061217	Technical Report	March 2015-May 2016

4. TITLE
INCORPORATION OF ANTIBIOTICS EFFECTIVE AGAINST MULTIDRUG-RESISTANT PATHOGENS INTO PMMA FOR CRANIO-MAXILLOFACIAL IMPLANTS

5a. Contract Number:
5b. Grant Number:
5c. Program Element Number:
5d. Project Number:
5e. Task Number:
5f. Work Unit Number: 1315

6. AUTHORS
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Naval Medical Research Unit San Antonio
3650 Chambers Pass, Bldg 3610
FT. Sam Houston, TX 78234

8. PERFORMING ORGANIZATION REPORT NUMBER
Tech Doc No.2018-02

9. SPONSORING/MONITORING AGENCY NAMES(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(s)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES
X **If this work is intended for submission to a journal, use the following statement:** This is a peer-reviewed journal submission. Do not cite, quote, or release until publication.

14. ABSTRACT

The rise in incidence of infections caused by multidrug-resistant (MDR) pathogens such as *Staphylococcus aureus* presents a problem in the treatment of post-surgical infections at cranio-maxillofacial implant sites. Incorporation of antibiotics against MDR pathogens into polymethyl methacrylate (PMMA) is a potential treatment to reduce infections. The objective of this study was to evaluate the structural, mechanical, and antimicrobial activity of PMMA incorporated with colistin, polymyxin B, or minocycline against Gram-positive *S. aureus* and Gram-negative *A. baumanniii*. Two ratios of antibiotic (0.5g/40 mL and 1.0g,/40 mL) were incorporated into PMMA test rods. All three antibiotics were successfully incorporated into PMMA at ratios of 0.5g and 1.0g of antibiotics. The addition of the antibiotics and immersion in water for 30 days did not significantly affect the structural or mechanical properties of the polymer. Each antibiotic exhibited a burst release profile, except for minocycline at 1.0g which showed a sustained release profile. However, addition of colistin at any concentration and of minocycline at higher concentrations may not be preferred for sustained release because the heterotrophic resistance properties of certain bacterial strains in response to these antibiotics may lead to adverse nosocomial infections.

15. SUBJECT TERMS
Acinetobacter baumannii, American Society for Testing and Materials, Colistin, Gas permeation chromatography, High performance liquid chromatography, Multidrug resistant, Methyl methacrylate, Polymyxin B, Polymethyl methacrylate, *Staphylococcus aureus.*

16. SECURITY CLASSIFICATION OF:	17. LIMITATION	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON

a. REPORT	b. ABSTRACT	c. THIS PAGE	OF ABSTRACT	OF PAGES	Commanding Officer
UNCL	UNCL	UNCL	UNCL	X	19b. TELEPHONE NUMBER (INCLUDING AREA CODE) COMM/DSN: 210-539-5334 (DSN: 389)